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(54) Title: TRANSGENIC PLANTS WITH TOCOPHEROL METHYLTRANSFERASE

(57) Abstract

Disclosed are gene sequences encoding γ–tocopherol methyltransferases from photosynthetic organisms. The enzyme γ–tocopherol methyltransferase catalyzes the methylation of γ–tocopherol to yield α–tocopherol, the most bioactive species of tocopherol. γ–Tocopherol methyltransferase is believed to be involved in regulating the relative amounts of the various tocopherols present in photosynthetic organisms. By introducing a genetic construct having a γ–tocopherol methyltransferase coding sequence placed under the control of a plant promoter into a plant, transgenic plants can be made having altered γ–tocopherol methyltransferase expression, to effect dramatic changes in the tocopherol profile of the plant. Transgenic plants can be made that have α–tocopherol as the predominant tocopherol in their seeds and oils.

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TRANSGENIC PLANTS WITH TOCOPHEROL METHYLTRANSFERASE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Serial No. 60/053,819 filed July 25, 1997 and U.S. Provisional Application Serial No. 60/072,497 filed January 26, 1998.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Vitamin E is an essential component of mammalian diets. Epidemiological evidence indicates that Vitamin E supplementation results in decreased risk for cardiovascular disease and cancer, aids in immune function, and generally prevents or slows a number of degenerative disease processes in humans (Traber and Sies, Annu. Rev. Nutr. 16:321-347, 1996). Vitamin E functions in stabilizing the lipid bilayer of biological membranes (Skrypin and Kagan, Biochim. Biophys. Acta 815:209 1995; Kagan, N.Y. Acad. Sci. p 121, 1989; Gomez-Fernandez et al., Ann. N.Y. Acad. Sci. p 109, 1989), reducing polyunsaturated fatty acid (PUFA) free radicals generated by lipid oxidation (Fukuzawa et al., Lipids 17: 511-513, 1982), and quenching singlet oxygen species (Fryer, Plant Cell Environ. 15(4):381-392, 1992).

Vitamin E, or α-tocopherol, belongs to a class of lipid-soluble antioxidants that includes α, β, γ, and δ-tocopherols and α, β, γ, and δ-tocotrienols. Although α, β, γ, and δ-tocopherols and α, β, γ, and δ-tocotrienols are sometimes referred to collectively as "Vitamin E" in the popular press, Vitamin E is properly defined chemically solely as α-tocopherol. Of the various tocopherols present in foodstuff, α-tocopherol is the most significant for human health both

because it is the most bioactive of the tocopherols and also because it is the tocopherol most readily absorbed and retained by the body (Traber and Sies, Annu. Rev. Nutr. 16:321-347, 1996). The *in vivo* antioxidant activity of α-tocopherol is higher than the antioxidant activities of β, γ, and δ-tocopherol (Kamal-Eldin and Appelqzvist Lipids 31:671-701, 1996).

Only plants and certain other photosynthetic organisms, including cyanobacteria, synthesize tocopherols. Therefore, dietary tocopherols are obtained almost exclusively from plants. Plant tissues vary considerably in total tocopherol content and tocopherol composition. The predominant tocopherol in green, photosynthetic plant tissues often is α-tocopherol. Leaf tissue can contain from 10-50 μg total tocopherols/gram fresh weight.

Non-green plant tissues and organs exhibit a wider range of both total tocopherol levels and tocopherol compositions. In general, most of the major food staple corps (e.g., rice, corn, wheat, potato) produce low to extremely low levels of total tocopherols, of which only a small percentage is α-tocopherol (Hess, Vitamin E, α-tocopherol, *In* Antioxidants in Higher Plants, R. Alscher and J. Hess, Eds. 1993, CRC Press, Boca Raton. pp 111-134). Oil seed crops generally contain much higher levels of total tocopherols; however, α-tocopherol is present only as a minor component and β, γ, and δ-tocopherols and tocotrienols predominate (Taylor and Barnes, Chemy Ind., Oct.:722-726, 1981).

Daily dietary intake of 15-30 mg of vitamin E is recommended to obtain optimal plasma α-tocopherol levels. It is quite difficult to achieve this level of vitamin E intake from the average American diet. For example, one could obtain the recommended daily dose of Vitamin E by daily consumption of over 750 grams of spinach leaves (in which α-tocopherol comprises 60% of total tocopherols) or 200-400 grams of soybean oil.

One alternative to relying on diet alone to obtain the recommended levels of vitamin E is to take a vitamin E

supplement. However, most vitamin E supplements are synthetic vitamin E having six stereoisomers, whereas natural vitamin E vitamin is a single isomer. Furthermore, supplements tend to be relatively expensive, and the general population is disinclined to take vitamin supplements on a regular basis.

Although tocopherol function in plants has been less extensively studied than tocopherol function in mammalian systems, it is likely that the analogous functions performed by tocopherols in animals also occur in plants. In general, plant tocopherol levels have been found to increase with increases in various stresses, especially oxidative stress. Increased α-tocopherol levels in crops are associated with enhanced stability and extended shelf life of fresh and processed plant products (Peterson, Cereal-Chem 72(1):21-24, 1995; Ball, Fat-soluble vitamin assays in food analysis. A comprehensive review. London: Elsevier Science Publishers LTD, 1988).

Vitamin E supplementation of swine, beef, and poultry feeds has been shown to significantly increase meat quality and extend the shelf life of post-processed meat products by retarding post-processing lipid oxidation, which contributes to the formation of undesirable flavor components (Ball, *supra* 1988; Sante and Lacourt, J. Sci. Food Agric. 65(4):503-507, 1994; Buckley et al., J. of Animal Science 73:3122-3130, 1995).

What would be useful for the art is a method to increase the ratio of α-tocopherol to γ-tocopherol in seeds, oils, and leaves from crop and forage plants, or a method for producing natural vitamin E in nonphotosynthetic bacteria or fungi using a large scale fermentation process. Increasing α-tocopherol levels in crop plants would increase the amount of α-tocopherol obtained in the human diet, and would enhance the stability and shelf life of plants and plant products. The meat industry would benefit from the development of forage plants having increased levels of vitamin E.

BRIEF SUMMARY OF THE INVENTION

The present invention is based on an isolated DNA fragment including a coding sequence for a γ-tocopherol

methyltransferase.

The invention is also a heterologous genetic construct comprising a γ-tocopherol methyltransferase coding sequence operably connected to a plant, bacterial, or fungal promoter not natively associated with the γ-tocopherol methyltransferase coding sequence.

Another aspect of the present invention is a method of altering the tocopherol profile of a plant comprising the steps of: (a) providing a heterologous genetic construct comprising a γ-tocopherol methyltransferase coding sequence operably connected to a plant promoter not natively associated with the coding sequence; and (b) introducing the construct into the genome of a plant.

The present invention is also directed toward transgenic plants which have an altered ratio of α-tocopherol to γ-tocopherol, thus increasing the nutritive value of the plants and products therefrom for human and animals.

In another embodiment, the invention is a plant comprising in its genome a heterologous genetic construct comprising a γ-tocopherol methyltransferase coding sequence operably connected to a promoter that is functional in plants.

It is an object of the present invention to provide a genetic construct comprising a coding sequence for a γ-tocopherol methyltransferase operably connected to a plant promoter not natively associated with the coding sequence which when expressed in a plant comprising the construct in its genome results in an alteration in the ratio of α-tocopherol:γ-tocopherol in the plant, relative to an untransformed wild-type plant.

It is an object of this invention to provide a plant having an altered α-tocopherol:γ-tocopherol ratio.

Other objects, features, and advantages of the invention will become apparent upon review of the specification and claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 shows the alignment of amino acid sequences of γ-tocopherol methyl-transferases from *Arabidopsis thaliana* and *Synechocystis*. Inverted triangles denote putative cleavage sites of N-terminal targeting domains; the closed circle denotes the position of an in-frame *NcoI* site in the leader peptide of SLR0089.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is, in part, directed to a plant comprising in its genome a genetic construct comprising a γ-tocopherol methyltransferase coding sequence operably connected to a plant promoter not natively associated with the coding sequence. Such transgenic plants exhibit an altered ratio relative to the wild type plants of the same species. In fact, seed and seed oil of a plant not normally containing α-tocopherol can be altered so that the most abundant tocopherol is α-tocopherol. Alternatively, the relative percentage of γ-tocopherol present in plant tissue may be increased by reducing the activity of γ-tocopherol methyltransferase in the plant, which could be accomplished by expression of a γ-tocopherol methyltransferase coding sequence in the antisense orientation. The development of plants with increased γ-tocopherol may be useful in certain industries.

Tocopherols and plastoquinones, the most abundant quinones in plant plastids, are synthesized by a common pathway (Hess, Antioxidants in Higher Plants, CRC Press: Boca Raton p 140-152, 1993; Soll, Plant Cell Membranes, Academic Press: San Diego p 383-392, 1987). The synthesis of tocopherols involves four steps catalyzed by at least six enzymatic activities. A branchpoint in the common pathway occurs upon phytylation or prenylation of the precursor homogentisic acid to form either 2-methyl-6-phytylplastoquinol or 2-methyl-6-solanylplastoquinol, intermediates in tocopherol and plastoquinone biosynthesis, respectively.

The intermediate 2-methyl-6-phytylplastoquinol is the common precursor to the biosynthesis of all tocopherols. In

spinach leaves, the intermediate undergoes ring methylation to yield 2,3-dimethyl-6-phytylplastoquinol, which is cyclized to form γ-tocopherol. A second ring methylation at position 5 yields α-tocopherol (Soll and Schultz, Phytochemistry 19(2):215-218, 1980). The second ring methylation is catalyzed by γ-tocopherol methyltransferase, a distinct enzymatic activity from the methyltransferase that catalyzes the methylation at position 7, and the only enzyme of the pathway that has been purified from plants (d'Harlingue and Camara, J. Biol. Chem. 260(68): 15200-15203, 1985; Ishiko et al., Phytochemistry 31(5):1499-1500, 1992).

The methylation enzymes are involved in regulating the final composition of the tocopherol pool. Data obtained in studies of sunflower mutants suggest that the enzymes involved in methylation have a high degree of influence over relative tocopherol amounts but do not affect the overall regulation of total tocopherol content(Demurin, Helia 16:59-62, 1993). Normally, seed tocopherol composition in cultivated sunflower (*Helianthus annuus* L.) is primarily α-tocopherol (i.e., 95-100% of the total tocopherol pool) (Skoric et al., Proceedings of the 14th International Sunflower Conference. 1996. Beijing/Shenyang, China). However, two mutant sunflower lines were identified with tocopherol compositions of 95% γ-tocopherol/5% α-tocopherol and 50% β-tocopherol/50% α-tocopherol. Although these presumed tocopherol methylation mutants were found to have dramatically different tocopherol profiles in seed, total tocopherol levels were not significantly different than those of wild type sunflower (Demurin, *supra* 1993). Based on these results, we hypothesized that it should be possible to alter the tocopherol profile of many plant species by manipulating γ-tocopherol methyltransferase expression without affecting the total tocopherol pool size.

The enzyme γ-tocopherol methyltransferase catalyzes the methylation of γ-tocopherol to form α-tocopherol, the final step in α-tocopherol biosynthesis. Overexpression of a γ-tocopherol methyltransferase gene in a plant enhanced the

conversion of γ-tocopherol to α-tocopherol in any tissue containing γ-tocopherol, thereby increasing the α-tocopherol:γ-tocopherol ratio. In fact, seed and oil in which little or no α-tocopherol is found can be altered to contain predominantly α-tocopherol. Conversely, expression of the antisense RNA would be expected to reduce expression of the γ-tocopherol methyltransferase, causing a decrease in the α-tocopherol:γ-tocopherol ratio. Plants having increased γ-tocopherol may be useful for certain industries.

We have discovered that γ-tocopherol methyltransferase also catalyzes the conversion of δ-tocopherol to β-tocopherol. Overexpression of γ-tocopherol methyltransferase in plant tissue results in increased conversion of δ-tocopherol to β-tocopherol. It is expected that expression of γ-tocopherol methyltransferase antisense RNA would result in reduced conversion of δ-tocopherol to β-tocopherol.

As demonstrated in the examples below, the seed of *Arabidopsis* plants transformed with a genetic construct comprising an Arabidopsis γ-tocopherol methyltransferase gene under the control of either the seed specific promoter or the constitutive cauliflower mosaic virus 35S promoter exhibit a dramatic increase in the ratio of α-tocopherol:γ-tocopherol. No α-tocopherol is detected in the seed of untransformed *Arabidopsis*, whereas seed from *Arabidopsis* transformed with the γ-tocopherol methyltransferase gene under the control of the seed-specific promoter contained about 90% α-tocopherol. Seed from *Arabidopsis* transformed with the γ-tocopherol methyltransferase gene under the control of a constitutive promoter contained slightly less α-tocopherol (84%). This observation demonstrates that for plants natively having a tocopherol profile in which α-tocopherol is not predominant (i.e. is less than 50% of total tocopherol), that α-tocopherol can be made to be the predominant tocopherol form in seed or seed oil from a transgenic plant.

Methylation of γ-tocopherol to form α-tocopherol is the means by which the ratio of the di-methylated tocopherols (γ-tocopherol) and tri-methylated tocopherol (α-tocopherol) is

regulated. By up regulating γ-tocopherol methyltransferase expression in tissues in which it is not normally expressed in a plant, it is now possible to increase α-tocopherol levels in tissues of many agricultural crops in which γ-tocopherol is a major tocopherol (e.g., maize, soybean, rapeseed, cotton, peanut, safflower, castor bean, rice). Many common edible seed oils have large amounts of γ-tocopherol. Increasing the level of expression of γ-tocopherol methyltransferase in seed oil plants should increase the ratio of α-tocopherol:γ-tocopherol.

Isolation and functional analysis of the γ-tocopherol methyltransferase genes from *Synechocystis* PCC6803 and *Arabidopsis thaliana* was accomplished by concurrently pursuing the complementary molecular genetic approaches described in detail in the examples. These two model organisms were selected because both synthesize tocopherols by similar or identical pathways and both are highly tractable genetic, molecular, and biochemical systems.

The DNA sequences of the γ-tocopherol methyltransferase genes from *Synechocystis* PCC6803 and *Arabidopsis thaliana* are shown in SEQ ID NO:1 and SEQ ID NO:3, respectively. The corresponding deduced amino acid sequences of the proteins are shown in SEQ ID NO: 2 and SEQ ID NO:4.

It is expected that the present invention may be practiced using a γ-tocopherol methyltransferase gene from any photosynthetic organism. It is well within the ability of one of skill in the art to isolate a plant γ-tocopherol methyltransferase gene using the sequences disclosed herein. The usefulness of these sequences to identify other γ-tocopherol methyltransferase coding sequences is demonstrated by the fact that it was the *Synechocystis* sequence that was used to identify the Arabidopsis sequence. The two sequences can be used to screen public computer databases of plant cDNAs (dbest databases) and genomic sequences. Alternatively, the sequences could be used to design probes for use in identifying genomic or cDNA clones containing a γ-tocopherol methyltransferase sequence. Another approach would be to use the sequences to design oligonucleotide primers for use in PCR

amplification of γ-tocopherol methyltransferase genes from plant DNA.

To determine whether one has identified a γ-tocopherol methyltransferase sequence, one could perform a gene replacement study using wild type *Synechocystis*, a complementation study using a *Synechocystis* γ-TMT knockout mutant, or an *in vitro* enzyme assay using a suitable substrate and γ-tocopherol methyltransferase protein expressed in *E. coli* or another suitable expression system. A genetic construct comprising the γ-tocopherol methyltransferase coding sequence operably connected to a plant promoter can be constructed and used to transform *Arabidopsis* or a plant or crop plant of interest. A transgenic plant comprising the construct in its genome would be expected to have altered expression of γ-tocopherol methyltransferase and an altered tocopherol profile relative to an untransformed, wild-type plant.

It is expected that polyploid plants having more than one copy of the γ-tocopherol methyltransferase gene may have allelic variations among γ-tocopherol methyltransferase gene sequences. It is anticipated that putative γ-tocopherol methyltransferase gene sequences having less than 100% homology to SEQ ID NO:1 or SEQ ID NO:3 encode proteins having γ-tocopherol methyltransferase activity.

It is envisioned that minor sequence variations from SEQ NO:1 or SEQ ID NO:3 associated with nucleotide additions, deletions, and mutations, whether naturally occurring or introduced *in vitro*, will not affect γ-tocopherol methyltransferase activity. The scope of the present invention is intended to encompass minor variations in γ-tocopherol methyltransferase sequences. Also, it is now well within the level of ordinary skill in the art of plant genetic engineering to alter the coding sequence for a gene by changing codons specifying for common amino acids or by making conservative amino acid substitutions at DNA sequences encoding non-critical portions of enzymes.

Construction of an expression vector comprising a γ-tocopherol methyltransferase coding sequence operably connected

to a plant promoter not natively associated with the coding sequence will be achieved using standard molecular biology techniques known to the art. The plant promoter may be a tissue-specific promoter such as a seed-specific promoter (e.g., napin or DC3), a constitutive promoter such as CaMV 35S, a developmental stage-specific promoter, or an inducible promoter. Promoters may also contain certain enhancer sequence elements that improve efficiency of transcription. Optionally, the construct may contain a termination signal, such as the nopaline synthase terminator (NOS). Preferably, the constructs will include a selectable or screenable marker to facilitate identification of transformants. The constructs may have the coding region in the sense or antisense orientation.

Once a genetic construct comprising a γ-tocopherol methyltransferase gene has been obtained, it can readily be introduced into a plant or plant tissue using standard methods known to the art. For example, the *Agrobacterium* transformation system is known to work well with all dicot plants and some monocots. Other methods of transformation equally useful in dicots and monocots may also be used. Transgenic plants may be obtained by particle bombardment, electroporation, or by any other method of transformation known to one skilled in the art of plant molecular biology. The experience to date in the technology of plant genetic engineering has taught that the method of gene introduction does not affect the phenotype achieved in the transgenic plants.

A transgenic plant may be obtained directly by transformation of a plant cell in culture, followed by regeneration of a plant. More practically, transgenic plants may be obtained from transgenic seeds set by parental transgenic plants. Transgenic plants pass on inserted genes, sometimes referred to as transgenes, to their progeny by normal Mendelian inheritance just as they do their native genes. Methods for breeding and regenerating plants of agronomic interest are known to the art. Experience with transgenic plants has also demonstrated that the inserted gene, or

transgene, can be readily transferred by conventional plant breeding techniques into any desired genetic background.

It is reasonable to expect that the expression of heterologous γ-tocopherol methyltransferase in a transgenic plant will result in alterations in the tocopherol profile in that plant. In addition to the inherent advantage of increasing the α-tocopherol:γ-tocopherol ratio, changes in the tocopherol profile may result in unique, advantageous phenotypes. This invention is intended to encompass other advantageous phenotypes that may result from alterations in tocopherol biosynthesis in plants obtained by the practice of this invention.

Using the information disclosed in this application and standard methods known to the art, one of skill in the art could practice this invention using any crop plant or forage plant of interest.

The following nonlimiting examples are intended to be purely illustrative.

EXAMPLES

Example 1. Identification and Characterization of a Putative γ-TMT Gene in *Synechocystis* PCC6803

We recently cloned and characterized the γ-tocopherol methyltransferase gene from *Synechocystis* as follows. An *Arabidopsis* p-hydroxyphenyl-pyruvic acid dehydrogenase (HPPDase) cDNA sequence (Norris and Della Penna, submitted, Genbank Accession # AF000228, Plant Physiol., in press) was used to search a database containing the DNA sequence of the *Synechocystis* PCC6803 genome (Kaneko et al., DNA Res. 3:109-136, 1996). We identified an open reading (designated SLR0090) that shares a high degree of amino acid sequence similarity (i.e. 35% identity and 61% similarity) with the *Arabidopsis* HPPDase enzyme. The putative *Synechocystis* HPPDase gene is located within an operon in the *Synechocystis* genome comprised of 10 open reading frames (ORFs) encompassing bases 2,893,184 to 2,905,235 of the published *Synechocystis* PCC6803 genome (Kaneko et al., *supra* 1996). We hypothesized that this operon

might also contain additional genes that encode other enzymes involved in tocopherol synthesis.

Two ORFs (SLR0089 and SLR0095) were identified as possible candidates for *Synechocystis* tocopherol methyltransferase genes. BLAST searches with ORFs SLR0089 and SLR0095 showed that these proteins share a high degree of similarity to the known protein sequences of Δ-(24)-sterol-C-methyltransferases and various plant caffeol CoA-O-methyltransferases, respectively. Both SLR0089 and SLR0095 proteins contain consensus sequences corresponding to conserved S-adenosyl-methionine (SAM) binding domains (Kagan and Clarke, Archives of Biochem. and Biophy. 310(2):417-427, 1996). The SLR0089 protein contains other structural features that are consistent with features found in a tocopherol methyltransferase. These features were not found in SLR0095. First, PSORT (Prediction of Protein Localization Sites) computer analysis of the two protein sequences predict that SLR0089 is localized to the plasma membrane, whereas and SLR0095 is localized to the cytosol. Tocopherol biosynthesis in cyanobacteria is believed to occur in the plasma membrane; therefore, localization of SLR0089 protein to the plasma membrane suggests that it may be a tocopherol methyltransferase. Additionally, PSORT analysis identified the presence of a putative bacterial signal sequence in the first 25 amino acids of the SLR0089 protein. The predicted molecular weight of the mature SLR0089 protein (after truncation of the signal sequence) is 32,766 daltons, which is very close to the reported molecular weight (33,000 daltons) of purified from pepper fruits (d'Harlingue and Camara, *supra* 1985). The predicted molecular weight of SLR0095 is 24,322 daltons. Therefore, we concluded that of the two identified ORFs, the SLR0089 gene was more likely to be a tocopherol methyltransferase.

Example 2. Amplification and cloning of the *Synechocystis* γ-TMT gene

Synechocystis genomic DNA was isolated by the method of Williams (Methods Enzymol.167:776-778, 1987). The SLR0089

gene was amplified from *Synechocystis* genomic DNA by polymerase chain reaction (PCR) using a sense strand specific primer(SLR0089F, SEQ ID NO:5) and a non-sense strand specific primer SLR0089R (SEQ ID NO:6) under the following conditions:

The amplification of the SLR0089 open reading frame was conducted in a 50μl reaction volume containing 0.4 mM dATP, 0.4 mM dGTP, 0.4 mM dCTP, 0.4 mM dCTP, 0.4 mM dTTP, 0.2 μM SLR0089F primer, 0.2 μM SLR0089R primer, 10 ng Synechocystis PCC6803 genomic DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, and 2.5 units *Taq* polymerase (Gibco-BRL). PCR thermocycle conditions were performed as follows:

5 minutes 95°C (1 cycle)
1 minute 95°C -> 1 minute 55°C -> 1.5 minutes 72°C (35 cycles)
7 minutes 72°C (1 cycle)

The PCR product comprising the SLR0089 ORF was cloned using standard molecular biological techniques known to one of skill in the art. Briefly, the amplified SLR0089 ORF was purified and made blunt ended by treatment with the Klenow fragment. The SLR0089 gene was ligated to *Eco*RV-linearized pBluescript KS II (Stratagene, Inc., LaJolla, CA). The ligation mixture was used to transform competent *E. coli* DH5α cells, and putative transformants were selected on the basis of ampicillin resistance. A plasmid designated pH-1 that was isolated from a transformant was found to contain the SLR0089 insert. The identity of the SLR0089 gene (SEQ ID NO:1) was confirmed by sequencing using T7 and T3 sequencing primers.

Example 3. Development of a SLR0089 knockout mutant

A gene replacement vector was constructed using standard molecular biology techniques. The plasmid pH1, which contains a unique NcoI site in the SLR0089 ORF, was digested with NcoI restriction endonuclease. The aminoglycoside 3'-phospho-transferase gene from Tn903 was ligated to the *Nco*I site of pH1 and the ligation mixture was used to transform *E. coli* DH5α cells. Transformants were selected using kanamycin and

ampicillin. A recombinant plasmid (pQ-1) containing the disrupted SLR0089 ORF was isolated and used to transform Synechocystis PCC6803 according to the method of Williams (Methods Enzymol.167:776-778, 1987).

Synechocystis transformants were selected for on BG-11 medium (Castenholz, Methods in Enzymology p 68-93, 1988) containing 15 mM glucose and 15 μg/ml kanamycin. All cultures were grown under continuous light at 26°C. Four independent transformants were carried through five subculturings of single colonies to fresh medium. PCR and genomic analysis were used to confirm that the gene replacement was successful and complete.

Example 4. Tocopherol profiles of wild type and mutant Synechocystis

Approximately 200 mg of cells were scraped from 2 week old *Synechocystis* cultures grown on BG-11 agar medium. The cells were homogenized in 6 ml of 2:1 (volume:volume) methanol:$CHCl_3$ containing 1 mg/ml butylated hydroxytolulene (BHT) using a polytron homogenizer. Following homogenization, 2 ml of $CHCl_3$ and 3.4 ml of double-distilled water was added to the homogenate. The lower lipid phase was removed and dried under nitrogen gas. The dried lipids were resuspended in 200μl of HPLC grade ethyl acetate containing 1 mg/ml BHT.

Tocopherols were analyzed by reverse phase HPLC using a Hewlett-Packard Series 1100 HPLC system with a fluorescence detector. Crude lipid extracts were fractionated on a Water Spherisorb S5 ODS2 4.6 X 250 mm column in a mobile phase consisting of 75% methanol and 25% isopropanol and a flow rate of 1 ml/min. The fluorescence was measured at 330 nm after excitation at a wavelength of 290 nm.

Wild-type *Synechocystis* produces α-tocopherol as its most abundant tocopherol (>95%of total tocopherols). The SLR0089 disrupted mutant of *Synechocystis* is no longer able to synthesize α-tocopherol and instead accumulates γ-tocopherol as its sole tocopherol. The elimination of α-tocopherol production and concomitant accumulation of γ-tocopherol

conclusively demonstrates that SLR0089 encodes γ-tocopherol methyltransferase, the final step in α-tocopherol biosynthesis.

Example 5. Identification of a Putative *Arabidopsis* γ-TMT cDNA from the EST Database

The *Arabidopsis* EST database (Ausbel et al., Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, N.Y., 1987) was searched using the *Synechocystis* γ-TMT DNA and protein sequences as queries. Two cDNA clones that share significant homology with the *Synechocystis* sequence were identified: the *Arabidopsis* Δ-(24)-sterol-C-methyltransferase and the *Arabidopsis* expressed sequence tag (EST) clone 165H5T7. Because the Δ-(24)-sterol-C-methyltransferase was functionally identified by its ability to complement a yeast Δ-(24)-sterol-C-methyltransferase mutant (*erg6*), we are confident that the clone does not encode a γ-TMT (Husselstein *et al.*, FEBS Letters 381:87-92, 1996). Therefore, we decided to focus our efforts on the *Arabidopsis* 165H5T7 EST clone (Genbank Accession #R30539). The DNA sequence of the 165H5T7 EST clone was determined (SEQ ID NO:3) and the amino acid sequence of the putative protein was deduced. The sequence was aligned with that of the *Synechocystis* γ-TMT (Fig 1). The full-length 165HT7 clone encodes a protein that is 35% identical and 66% similar to the Synechocystis γ-TMT and exhibits large blocks of identity. When 165H5T7 was used as query against the non-repetitive protein database, it was found to have the highest homology to SLR0089 ($P<10^{-54}$) and only moderate homology to the four known plant Δ-(24)-sterol-C-methyltransferases ($P\geq10^{-5}$). 165H5T7 also contains conserved SAM binding motifs common to a large number of methyltransferases (Fig. 1) but lacks proposed sterol binding domains common in the four plant Δ-(24)-sterol-C-methyltransferases identified to date (Husselstein *et al.*, *supra* 1990). These data suggest that clone 165H5T7 encodes an *Arabidopsis* γ-TMT homologue, which we have designated A.t.γ-TMT.

Example 6. Characterization of the putative *Arabidopsis* γ-TMT homologue using the gene replacement in *Synechocystis*

Plant cDNAs encoding putative γ-TMT homologues may be functionally identified using one of two gene replacement approaches in *Synechocystis*. One approach that may be employed is to replace the endogenous *Synechocystis* γ-TMT gene in wild type *Synechocystis* with the putative Arabidopsis γ-TMT cDNA 165H7T7. A *Synechocystis* γ-TMT(coding sequence # SLR0089) gene replacement vector will be constructed to include the following features, in 5' to 3' order: 1) at least 300 base pairs of DNA sequence corresponding to the *Synechocystis* genomic sequence found immediately upstream (5') of the native SLR0089 gene; 2) the first 77 base pairs of the SLR0089 ORF corresponding to the identified bacterial signal sequence that ends with a unique, in-frame *Nco*I site; 3) a polylinker or multiple cloning site; 4) an antibiotic resistance marker (e.g., a kanamycin resistance gene cassette); and 5) at least 300 base pairs of DNA sequence corresponding to the *Synechocystis* genomic sequence found immediately downstream (3') of the native SLR0089 gene. The putative plant γ-TMT cDNA to be tested for complementation will be inserted into the NcoI site or into the multiple cloning site.

The 165H5T7 cDNA may be engineered to contain an *Nco*I site at the transit peptide cleavage site predicted by PSORT using PCR mutagenesis, which would change the amino acid Val-48 to Met. The cDNA owill be ligated to the unique *Nco*I site in the SLR0089 gene replacement plasmid to create an in-frame, amino-terminal fusion between the *Synechocystis* γ-TMT signal peptide and the plant protein sequence. The construct will be used to transform wild type *Synechocystis;* transformants will be identified by kanamycin selection. After several single colony passages under selection, gene replacement will be confirmed by PCR. The tocopherol profile of transformants will be determined by HPLC. *Synechocystis* transformants functionally expressing *Arabidopsis* γ-TMT genes will be identified by their ability to synthesize α-tocopherol in the absence of a

functional *Synechocystis* γ-TMT gene.

In an alternative approach, the putative γ-TMT gene may be characterized according to its ability to complement the *Synechocystis* γ-TMT knockout mutant. The replacement vector could be constructed to include the intact putative γ-TMT gene and an antibiotic resistance marker other than kanamycin. Following transformation and selection, gene replacement can be confirmed by PCR and the transformants may be further characterized by tocopherol analysis.

Example 7. Functional characterization of *Arabidopsis* and *Synechocystis* γ-TMT genes by expression in *E. coli*

The proteins encoded by the *Synechocystis* SLR0089 gene and the *Arabidopsis* 165h5T7 cDNA clone were identified as γ-TMTs through functional expression in E. coli.

The SLR0089 gene was amplified from the *Synechocystis* PCC6803 genome using polymerase chain reaction (PCR). The forward primer (SLR0089coliF, SEQ ID NO:7), was designed to add a *Bsp*HI site to the 5' end of the primer. The reverse (3') PCR primer (SLR0089coliR, SEQ ID NO:8) was designed with a *Bgl*II site engineered at the 5' end of the primer.

The PCR reaction was conducted in two 100-μl reaction mixtures, each of which contained dNTPs (0.4 mM each), 2 μM SLR0089coliF, 2 μM SLR0089coliR, 10 ng *Synechocystis* PCC6803 genomic DNA, 10 mM KCl, 6.0 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.2), 2 mM $MgCl_2$, 0.1% Triton X-100, 10 μg/ml BSA, 2.5 units Pfu polymerase (Stratagene, LaJolla, CA). The following thermocycle conditions were used:

5 minutes 95°C (1 cycle)
0.75 minutes 94°C -> 0.75 minutes 55°C -> 2 minutes 72°C (30 cycles)
10 minutes 72°C (1 cycle)

The PCR fragment was gel-purified and ligated to *Eco*RV-linearized pBluescript KS II (Stratagene, LaJolla, CA). The ligation product was used to transform *E. coli* strain DH5α, and putative transformants were selected on the basis of ampicillin resistance. A recombinant plasmid containing the insert (designated p082297) was sequenced to confirm the correct amplification and subcloning of the SLR0089 sequence.

The deduced amino acid sequence of SLR0089 contains a putative amino-terminal bacterial signal sequence comprising the first 24 amino acids of the deduced amino acid sequence. Because this amino-terminal signal sequence could effect the conformation of the SLR0089 protein when expressed in *E. coli* and render the protein inactive, we modified the SLR0089 DNA sequence such that it encodes a truncated protein devoid of the putative amino-terminal bacterial signal sequence. The SLR0089 gene contains a *Nco*I recognition sequence at the predicted cleavage site for the putative bacterial signal sequence. A *Nco*I-*Bgl*II fragment containing a truncated SLR0089 DNA sequence from p082297-coli was subcloned in the correct reading frame into the *Nco*I and *Bam*HI sites of the T7 *E. coli* pET3D expression vector (Novagen, Madison, WI). The ligation mixture was used to transform *E. coli* BL21 (DE3) and transformants were selected for on the basis of ampicillin resistance. A plasmid (designated p011698-1) containing the insert was identified by restriction digest analysis with the enzyme *Hind*III.

The 165H5T7 cDNA clone was also subcloned into the pET3D expression vector. The first 50 N-terminal amino acids of the deduced amino acid sequence of 165H5T7 contains a putative amino-terminal chloroplast targeting sequence that could effect the conformation of the 165H5T7 protein when expressed in E. coli and render the protein inactive. Therefore, we modified the 165H5T7 DNA sequence to encode a truncated protein devoid of the putative amino-terminal chloroplast targeting sequence. The truncated 165H5T7 DNA sequence was obtained by PCR amplification of 165H5T7 cDNA using primers designed to amplify the sequence corresponding to the region between nucleotide 353 and nucleotide 1790 of the original 165H5T7 sequence. The

forward PCR primer (165matF, SEQ ID NO:9) adds a *Nco*I site to the 5' end of the truncated 165H5T7 sequence to facilitate cloning into the pET3D vector. The reverse (3') PCR primer (165matR, SEQ ID NO:10) was designed from the polylinker region of the pSPORT1 vector with a *Acc*I site engineered at the 5' end of the primer. The PCR reaction was conducted with the 165matF and 165matR primers (2μM each) using the same PCR conditions described for the amplification of the truncated Synechocystis gene, above.

Following gel purification, the PCR fragment was ligated to EcoRV-linearized pBluescript KS II, the ligation product was used to transform *E. coli* strain DH5α, and ampicillin-resistant putative transformants were selected. A recombinant plasmid (designated p010498-2) containing the insert was identified. The DNA sequence of p010498-2 was determined to confirm the correct amplification and subcloning of the truncated 165H5T7 sequence. The truncated 165H5T7 DNA sequence was subcloned as a *Nco*I-*Bam*HI fragment pET3D vector digested with *Nco*1 and *Bam*HI. The ligation product was used to transformed *E. coli* DH5α and transformants were selected for on the basis of ampicillin resistance. A plasmid (designated p011898-1) containing the insert was identified by restriction digest analysis with the enzyme *Hind*III.

The p011698-1 and p011898-1 constructs were used to transform the *E. coli* T7 expression host BL21(DE3). To generate protein for γ-TMT assays, one liter cultures of transformed host cells containing one of the constructs were grown in Luria broth containing 100 mg/liter ampicillin. Each culture was started at an optical density at 600 nm (OD_{600}) of 0.1 and incubated in a shaking incubator at 28°C until the culture reached an OD_{600} of 0.6, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to each culture to obtain a final concentration of 0.4 mM IPTG. Each culture was incubated for an additional 3 hours at 28°C and the cells were harvested by centrifugation at 8,000 g. The cell pellets were then resupended in 10 ml of 10 mM HEPES (pH 7.8), 5 mM DTT, 0.24 M sorbitol, 1 mM PMSF. The cells were lysed by sonication

with a micro-tip sonicator using four 10-second pulses. Triton X 100 was added to each homogenate to a final concentration of 1%. The homogenates were incubated on ice for 30 minutes, and subjected to centrifugation at 30,000 g for 30 minutes at 4°C. The supernatants of these extracts were assayed for γ-tocopherol methyltransferase activity as follows.

The γ-TMT assays were performed in 250 μl volumes containing 50 mM Tris (pH 7.5 for the *Synechocystis* and pH 8.5 for the *Arabidopsis* enzyme), 5 mM DTT, 5 mM γ- or δ-tocopherol, and 0.025 μCi (55μCi/mmole) (^{14}C-methyl)-S-adenosylmethionine. Reaction mixtures were incubated at room temperature for 30 minutes. The reactions were stopped by adding of 1 ml of 2:1 (v:v) $CHCl_3$:methanol containing 1 mg/ml butylated hydroxytolulene (BHT) and 250 μl of 0.9% NaCl in water, and vortexing. The samples were centrifuged to separate the phases. The $CHCl_3$ (lower) phase was transferred to a fresh tube containing 100 mg of α-tocopherol and the $CHCl_3$ was then removed under vacuum in a speed-vac. The dried lipid fraction was resuspended in 50 μl ethyl acetate containing 1 mg/ml BHT. The lipid extracts were fractionated on silica 60 TLC plates in dichloromethane. Tocopherols were then identified by co-migration with authentic tocopherol standards after staining the plate with Emmerie-Engels solution (0.1% FeCl3, 0.25% 2,2'-dipyridyl in ethanol). The band corresponding to α-tocopherol was scraped from the TLC plate and the amount of radioactive material present was determined by scintillation counting. These experiments showed that the proteins encoded by the *Synechocystis* SLR0089 and *Arabidopsis* 165H5T7 DNA sequences were able to convert γ-tocopherol to α-tocopherol.

The *Synechocystis* and *Arabidopsis* γ-tocopherol methyltransferases were tested for activity using several different methyl-substituted tocopherol substrates. Both enzymes were able to specifically convert δ-tocopherol to β-tocopherol. The two enzymes were unable to use tocol, 5,7-diemethyltocol, β-tocopherol, and γ-tocotrienol as substrates. These results indicate that both the *Synechocystis* and *Arabidopsis* γ-tocopherol methyltransferases catalyze the

methylation of carbon 5 of the tocopherol chromanol ring. The *Synechocystis* and *Arabidopsis* γ-TMTs appear to require substrates with a methyl-group present on the 8 position of the chromanol ring and a fully saturated prenyl-tail for activity. Our results indicate that *Arabidopsis* γ-TMT exhibits greater activity with γ-tocopherol as the substrate than with the δ-tocopherol substrate, whereas the *Synechocystis* γ-TMT appears to be equally active toward γ-tocopherol and δ-tocopherol.

Example 8. Qualitative manipulation of tocopherols in Arabidopsis and other plants by over expressing the Arabidopsis γ-tocopherol methyltransferase.

The results from HPLC analysis of lipid extracts made from *Arabidopsis* leaves and seeds indicate that these tissues have relatively simple tocopherol profiles. In *Arabidopsis* leaves, α-tocopherol is present at ~90% of the total tocopherol content, with γ-tocopherol comprising the remainder of the tocopherol content. In *Arabidopsis* seeds, γ-tocopherol is present at ~95% of the total tocopherol content in Arabidopsis seeds with the remaining 5% being composed of δ-tocopherol. These simple tocopherol profiles make *Arabidopsis* seed and leaf tissue ideal targets for evaluating the functional consequences of altering the expression of a γ-tocopherol methyltransferase gene in plants.

We hypothesized that increasing the expression of a γ-tocopherol methyltransferase gene in *Arabidopsis* would increase α-tocopherol levels as a proportion of the total tocopherols. To test this hypothesis, the full-length *Arabidopsis* γ-tocopherol methyltransferase cDNA clone 165H5T7 was over-expressed under the control of the strong constitutive cauliflower mosaic virus 35S transcript (CaMV 35S) promoter and the embryo-specific carrot DC3 promoter (Seffens WS *et al.*, Dev. Genet. 11: 65-76,1990) in transgenic *Arabidopsis*.

The seed-specific plant gene expression plasmid was constructed from a derivative of the *Agrobacterium* plant transformation vector, pBIB-Hyg (Becker, D. Nucleic Acids Res.

18:203, 1990). The carrot embryo DC3 promoter was isolated from the plasmid pBS-DC3 5' PH after digestion with *Hind*III and *Bam*HI. The DC3 *Hind*III and *Bam*HI promoter fragment was then treated with DNA polymerase to fill in the 5' over-hanging ends. The pBIB-Hyg plasmid was digested with *Hind*III and then treated with DNA polymerase to fill-in the 5' over-hanging ends. The DC3 promoter fragment was ligated to pBIB-Hyg to create a plasmid designated p111397. The *Arabidopsis* γ-tocopherol methyltransferase cDNA 165H5T7 was subcloned in the sense orientation as a *Sal*I-*Xba*I fragment into the *Sal*I and *Xba*I sites of p111397 to obtain p122997. The p122997 plasmid has the following features: 1) plant hygromycin selectable marker; 2) *Agrobacterium* T-DNA left and right border sequences; 3) the *Arabidopsis* 165H5T7 γ-tocopherol methyltransferase cDNA cloned between the carrot seed specific DC3 promoter and the nopoline synthase 3' transcriptional termination sequences; 4) the RK2 broad host bacterial plasmid origin of replication; and 5) bacterial kanamycin resistance selectable marker.

The constitutive *Arabidopsis* γ-tocopherol methyltransferase gene expression plasmid was derived from pSN506 CaMV 35S binary plant expression vector, a pART27 derivative in which the p-hydroxyphenol pyruvic acid dioxygenase (HPPDase) cDNA is under the control of the CaMV 35S promoter. (Norris and Della Penna, in press). The CaMV 35S/γ-tocopherol methyltransferase construct was made by replacing the HPPDase cDNA with the full length 165H5T7 cDNA sequence. The HPPDase cDNA fragment was removed from pSN506 by digesting the plasmid with *Xba*I and *Xho*I. The 5' DNA over-hanging ends of the pSN506 *Xba*I-*Xho*I vector fragment were filled in using the Klenow fragment of the *E. coli* DNA polymerase. The linearized vector was ligated to a blunt-ended *Xba*I-*Sal*I fragment from 165H5T7 encoding the full length γ-tocopherol methyltransferase. A recombinant plasmid containing the insert was obtained and designated p010398. The plasmid p010398 contains the following characteristics: 1) plant kanamycin selectable marker; 2) agrobacterium T-DNA left and right border sequences; 3) the Arabidopsis 165H5T7 γ-tocopherol

methyltransferase cDNA cloned between the CaMV 35S promoter and the nopoline synthase 3' transcriptional termination sequences; 4) the RK2 broad host bacterial plasmid origin of replication; and 5) bacterial kanamycin resistance selectable marker.

The constitutive and seed specific γ-tocopherol methyltransferase plant gene expression constructs (p122997 and p010398) and the appropriate empty vector control vectors (pART27 and p111397) were used to transform *Agrobacterium tumefaciens* strain C58 GV3101. Wild type *Arabidopsis* (ecotype Columbia) plants were transformed with these Agrobacterium strains using the vacuum infiltration method (Bechtold N, Ellis J, Pelletier G, in planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. CR Acad Sci Paris, 1993. 1144(2): 204-212). Seeds from the primary transformants were selected for resistance to the appropriate antibiotic on medium containing MS salts, 1% sucrose, 0.7% agar, and suitable levels of the antibiotic. Antibiotic resistant seedlings (representing the T1 generation) were transferred to soil and grown to maturity. Leaf and seed material from these T1 generation plants were analyzed by HPLC.

Example 9. Characterization of Transgenic Plants.

A. Analysis of transgenic Arabidopsis Tocopherol Profiles

Known weights of approximately 5 mg of plant material (i.e. seed or leaf) and 100 ng of tocol (for use as an internal standard) were homogenized in 300 μl of 2:1 (V/V) methanol: $CHCl_3$ containing 1 mg/ml butylated hydroxytolulene (BHT). One hundred μl of $CHCl_3$ and 180 μl of 0.9% (w/v) NaCl in water were added to the homogenate and the mixture was briefly vortexed. The mixture was then centrifuged and the lower ($CHCl_3$) fraction was removed and transferred to a fresh tube. The $CHCl_3$ fraction was dried under vacuum and the resulting lipid residue was resuspended in 100 μl of ethyl acetate for analysis by C18 reverse phase HPLC or in 100 μl of hexane for analysis by normal phase HPLC.

Crude lipid extracts were analyzed by normal phase or reverse phase HPLC for changes in tocoperhol profiles. Individual tocopherol species were quantified by comparing their fluorescence signals with standard curves made from known quantities of authentic tocopherol standards. Reverse phase HPLC was done as describe in example 4. Normal phase HPLC analysis was done on a Licosorb Si60A 4.6 X 250 mm HPLC column using the following conditions:
Column temperature: 42°C

mobile phase: solvent A = HPLC grade hexane
solvent B = diisopropylether

Gradient : (ml/min)	time	%solvent A	% solvent B	flow rate
	0	92%	8%	1
	20	82%	18%	1
	25	82%	18%	1
	25	92%	8%	2
	34	92%	8%	2

Fluorescence Detector Settings:
excitation wavelength: 290 nm
emmission wavelentgh: 325nm

The concentrations of the various tocopherol species obtained by HPLC analysis of T1 seed material from *Arabidopsis* plants transformed with p122997, p010398, p111398, pART27 are shown in Table 1. Plants over-expressing the γ-tocopherol methyltransferase using either the CaMV 35S or carrot DC3 promoters are able to convert the majority of the γ-tocopherol normally present in *Arabidopsis* seeds to α-tocopherol and also are able to convert the majority of the δ-tocopherol normally present in *Arabidopsis* seeds to β-tocopherol. These results show that γ-tocopherol methyltransferase activity is normally limiting in *Arabidopsis* seeds.

B. Analysis of γ-tocopherol methyltransferase activity in transgenic Arabidopsis seed

Seeds from the T1 generation plants transformed with p122997, p010398, p111397, and pART27 were assayed for γ-tocopherol methyltransferase activity. Protein extracts were made by homogenizing approximately 10 mg of seeds in 200 μl of 50 mM Tris pH 8.5, 5 mM DTT, 1% Triton X 100, 1 mM PMSF. The extracts were centrifuged for 5 minutes to remove insoluble material. A 25-μl aliquot of each extract supernatant was assayed for γ-tocopherol methyltransferase activity as described in example 7. No γ-tocopherol methyltransferase activity was detected in wild type seeds and empty vector controls. Activity in seed-specific lines was approximately 2 pmol/hr/mg protein, and in 35S constitutive expression lines activity was 0.5 pmol/hr/mg protein.

Example 11. Other Transgenic Plants.

Based on this data demonstrating that a simple insert of a α-tocopherol methyl transferase gene into a plant can dramatically change the relative proportions of tocopherols in a plant seed, it becomes possible to reasonably suggest the similar results that can be obtained in other plant species.

It is expected that one may manipulate tocopherol profiles in any plant species using the methods disclosed in the examples. Based on the concentration of the various tocopherols in untransformed plant tissue, we have predicted tocopherol profiles obtainable for a variety of plant tissue (Table 2). Note that several common plant oils (e.g. soybean) which are predominantly γ-tocopherol and contain low levels of α-tocopherol can be altered to be predominantly α-tocopherol.

All publications cited in this patent application are incorporated by reference herein.

The present invention is not limited to the exemplified embodiment, but is intended to encompass all such modifications and variations as come within the scope of the following claims.

Table 1

	ng α-tocopherol/ mg seed (% total tocopherol)	ng β-tocopherol/ mg seed (% total tocopherol)	ng γ-tocopherol/ mg seed (% total tocopherol)	ng δ-tocopherol/ mg seed (% total tocopherol)	ng total-tocopherol/mg seed (% total tocopherol)
122997-1 (seed specific promoter/ Arabidopsis γ-TMT)	523.28 ± 45.06 (88.91%)	23.91 ± 3.81 (4.06%)	41.38 ± 4.05 (7.03%)	ND (0%)	588.55 ± 48.02 (100%)
111397-2 (seed specific promoter /empty vector control)	ND (0%)	ND (0%)	409.16 ± 6.82 (95.11%)	17.81 ± 0.82 (4.89%)	430.19 ± 7.05 (100%)
010398-1 (constitutive promoter/ Arabidopsis γ-TMT)	373.85 ± 15.25 (83.74%)	17.16 ± 0.87 (3.84%)	55.41 ± 5.12 (12.41%)	ND (0%)	446.43 ± 18.46 (100%)
ART27-1 (constitutive promoter/empty vector control)	ND (0%)	ND (0%)	409.99 ± 7.00 (96.41%)	15.41 ± 0.11 (3.62%)	425.28 ± 6.80 (88.91%)

ND= none detected
All samples were analyzed in triplicate

Table 2

Crop Species (tissue)	Tocopherol composition of untransformed plant	Expected tocopherol composition of transgenic plants with γ-TMT over-expressed
Soybean[1] (seed/oil)	70% γ-tocopherol 22% δ-tocopherol 7% α-tocopherol 1% β-tocopherol	77% α-tocopherol 23% β-tocopherol
Oil Palm[1] (seed/oil)	25% α-tocopherol 30% α-tocotrienol 40% γ-tocotrienol 5% δ-tocotrienol	25% α-tocopherol 70% α-tocotrienol 5% β-tocotrienol
Peanut[2] (raw nut)	50% α-tocopherol 50% γ-tocopherol	100% α-tocopherol
Peanut[2] (nut oil)	33% α-tocopherol 66% γ-tocopherol	100% α-tocopherol
Safflower[2] (seed oil)	48% α-tocopherol 22% γ-tocopherol 30% δ-tocopherol	70% α-tocopherol 30% β-tocopherol
Rapeseed[2] (seed oil)	25% α-tocopherol 75% δ-tocopherol	100% α-tocopherol
Cotton Seed[1] (seed oil)	40% α-tocopherol 58% γ-tocopherol 2% δ-tocopherol	98% α-tocopherol 2% β-tocopherol
Wheat[2] (whole wheat flour)	20% α-tocopherol 7% α-tocotrienol 17% β-tocopherol 56% β-tocotrienol	20% α-tocopherol 7% α-tocotrienol 17% β-tocopherol 56% β-tocotrienol
Wheat[1] (germ oil)	75% α-tocopherol 25% γ-tocopherol	100% α-tocopherol
Corn[1] (oil)	22% α-tocopherol 68% γ-tocopherol 3% β-tocopherol 7% δ-tocopherol	90% α-tocopherol 10% β-tocopherol
Castor Bean[2] (oil)	50% γ-tocopherol 50% δ-tocopherol	50% α-tocopherol 50% β-tocopherol
Corn[2] (whole grain)	11% α-tocopherol 69% γ-tocopherol 4% α-tocotrienol 9% γ-tocotrienol 7% β-tocotrienol	80% α-tocopherol 13% α-tocotrienol 7% β-tocotrienol
Barley[2] (whole grain)	14% α-tocopherol 2% γ-tocopherol 10% β-tocopherol 44% α-tocotrienol 7% γ-tocotrienol 23% β-tocotrienol	16% α-tocopherol 10% β-tocopherol 51% α-tocotrienol 23% β-tocotrienol
Rice[2] (whole grain)	50% α-tocopherol 50% γ-tocopherol	100% α-tocopherol
Potato[2] (tuber)	95% α-tocopherol 5% γ-tocopherol	100% α-tocopherol

Sunflower[2] (seeds raw)	95% α-tocopherol 5% γ-tocopherol	100% α-tocopherol
Sunflower[1] (seed oil)	96% α-tocopherol 2% γ-tocopherol 2% β-tocopherol	98% α-tocopherol 2% β-tocopherol
Banana[1] (fruit)	100% α-tocopherol	100% α-tocopherol
Lettuce[1] (leaf)	53% α-tocopherol 47% γ-tocopherol	100% α-tocopherol
Broccoli[2]	72% α-tocopherol 28% γ-tocopherol	100% α-tocopherol
Cauliflower[2]	44% α-tocopherol 66% γ-tocopherol	100% α-tocopherol
Cabbage[1]	100% α-tocopherol	100% α-tocopherol
Apple[2]	100% α-tocopherol	100% α-tocopherol
Pears[2]	93% α-tocopherol 7% γ-tocopherol	100% α-tocopherol
Carrots[2]	94% α-tocopherol 4% γ-tocopherol 2% δ-tocopherol	98% α-tocopherol 2% β-tocopherol

[1]McLaughlin, P.J, Weihrauch, J.C. "Vitamin E content of foods", J. Am. Diet Ass. 75:647-665 (1979).
[2]Bauernfeind, J. "Tocopherols in foods", In Vitamin E: A Comprehensive Treatise, L.J Machlin ed., Marcel Dekker, Inc. New York pp 99-168.

CLAIMS

We claim:

1. An isolated DNA fragment comprising a γ-tocopherol methyltransferase coding sequence.

2. The DNA fragment of claim 1, wherein the fragment is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

3. An isolated DNA fragment comprising *Arabidopsis* γ-tocopherol methyltransferase.

4. An isolated DNA fragment comprising *Synechocystis* γ-tocopherol methyltransferase.

5. A genetic construct comprising a γ-tocopherol methyltransferase coding sequence operably connected to a plant promoter not natively associated with the coding sequence.

6. A genetic construct as claimed in claim 5, wherein the γ-tocopherol methyltransferase coding sequence is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

7. A transgenic plant comprising in its genome the genetic construct of claim 5.

8. The plant of claim 5, wherein the plant has an altered α-tocopherol:γ-tocopherol ratio relative to an untransformed wild-type plant.

9. The seed of the plant of claim 8.

10. The plant of claim 5, wherein the plant has an altered δ-tocopherol:β-tocopherol ratio relative to an untransformed wild-type plant.

11. The seed of the plant of claim 10.

12. Oil from the seed of claim 11.

13. A transgenic plant of a species in which natively α-tocopherol is not the predominant tocopherol in its seeds, the transgenic plant altered to produce α-tocopherol as the most abundant tocopherol in the seeds of the plant.

14. Seeds of the plant of claim 13.

15. Oil from the seeds of claim 14.

16. A transgenic plant as claimed in claim 13 wherein the transgenic plant carries in its genome a foreign genetic construction comprising a γ-tocopherol methyltransferase gene selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

17. A transgenic plant which has an altered profile of tocopherols in its seeds or oils compared to non-transgenic plants of the same species.

18. Seed of the plant of claim 17.

19. Oil from the seeds of claim 18.

20. A transgenic plant seed of a plant species in which α-tocopherol is natively not the predominant tocopherol in seeds, the transgenic plant seed containing α-tocopherol as the most abundant tocopherol present in the transgenic plant seed.

21. Oil from the seed of claim 20.

22. A transgenic plant having an altered relative proportion of tocopherols in its tissues as compared to non-transgenic plants of the same species, the transgenic plant comprising in its genome an inserted γ-tocopherol methyltransferase coding sequence.

23. The plant of claim 22 wherein the γ-tocopherol methyltransferase is in the sense orientation.

24. The plant of claim 22 wherein the γ-tocopherol methyltransferase is in its antisense orientation.

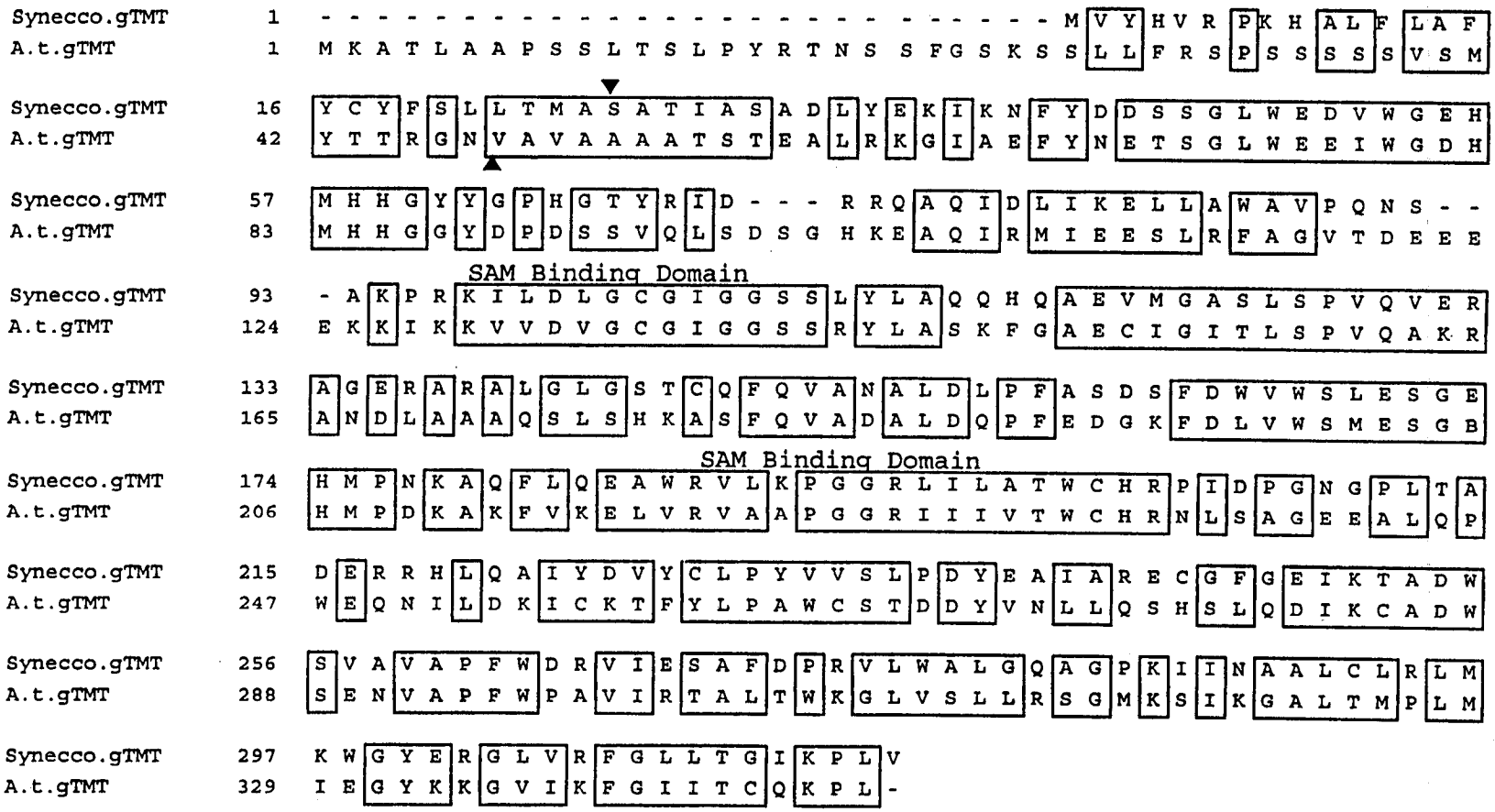
25. A method of producing α-tocopherol comprising the steps of:

(a) providing an expression host cell comprising in its genome a γ-tocopherol methyltransferase coding sequence operably connected to a promoter not natively associated with the sequence, wherein the promoter is functional in the host cell;

(b) culturing the host cell under conditions suitable to allow expression of the γ-tocopherol methyltransferase; and

(c) reacting γ-tocopherol and S-adenosylmethionine with the γ-tocopherol methyltransferase protein of step b under suitable conditions and for a period of time sufficient to allow conversion of γ-tocopherol to α-tocopherol.

SUBSTITUTE SHEET (RULE 26)


Synecco.gTMT 1 - - -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: DellaPenna, Dean
Shintani, David K.

(ii) TITLE OF INVENTION: TRANSGENIC PLANTS WITH TOCOPHEROL METHYLTRANSFERASE

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Quarles & Brady
(B) STREET: 1 South Pinckney Street
(C) CITY: Madison
(D) STATE: WI
(E) COUNTRY: US
(F) ZIP: 53701-2113

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Seay, Nicholas J.
(B) REGISTRATION NUMBER: 27386
(C) REFERENCE/DOCKET NUMBER: 920905.90024

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 608-251-5000
(B) TELEFAX: 608-251-9166

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 954 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..954

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATG GTT TAC CAT GTT AGG CCT AAG CAC GCC CTG TTC TTA GCA TTC TAT      48
Met Val Tyr His Val Arg Pro Lys His Ala Leu Phe Leu Ala Phe Tyr
 1               5                  10                  15
```

```
TGT TAT TTC TCT TTG CTT ACC ATG GCC AGC GCC ACC ATT GCC AGT GCA          96
Cys Tyr Phe Ser Leu Leu Thr Met Ala Ser Ala Thr Ile Ala Ser Ala
                20                  25                  30

GAC CTC TAC GAA AAA ATT AAA AAT TTC TAC GAC GAC TCC AGC GGT CTC         144
Asp Leu Tyr Glu Lys Ile Lys Asn Phe Tyr Asp Asp Ser Ser Gly Leu
            35                  40                  45

TGG GAA GAC GTT TGG GGT GAG CAT ATG CAC CAC GGC TAC TAC GGT CCC         192
Trp Glu Asp Val Trp Gly Glu His Met His His Gly Tyr Tyr Gly Pro
        50                  55                  60

CAC GGC ACC TAT CGG ATC GAT CGC CGC CAG GCT CAA ATT GAT CTG ATC         240
His Gly Thr Tyr Arg Ile Asp Arg Arg Gln Ala Gln Ile Asp Leu Ile
    65                  70                  75                  80

AAA GAA CTA TTG GCC TGG GCA GTG CCC CAA AAT AGC GCC AAA CCA CGA         288
Lys Glu Leu Leu Ala Trp Ala Val Pro Gln Asn Ser Ala Lys Pro Arg
                85                  90                  95

AAA ATT CTC GAT TTA GGC TGT GGC ATT GGC GGC AGT AGT TTG TAC TTG         336
Lys Ile Leu Asp Leu Gly Cys Gly Ile Gly Gly Ser Ser Leu Tyr Leu
            100                 105                 110

GCC CAG CAA CAC CAA GCA GAA GTG ATG GGG GCT AGT CTT TCC CCA GTG         384
Ala Gln Gln His Gln Ala Glu Val Met Gly Ala Ser Leu Ser Pro Val
        115                 120                 125

CAG GTG GAA CGG GCG GGG GAA AGG GCC AGG GCC CTG GGG TTG GGC TCA         432
Gln Val Glu Arg Ala Gly Glu Arg Ala Arg Ala Leu Gly Leu Gly Ser
    130                 135                 140

ACC TGC CAG TTT CAG GTG GCC AAT GCC TTG GAT TTG CCC TTT GCT TCC         480
Thr Cys Gln Phe Gln Val Ala Asn Ala Leu Asp Leu Pro Phe Ala Ser
145                 150                 155                 160

GAT TCC TTT GAC TGG GTT TGG TCG TTG GAA AGT GGG GAG CAC ATG CCC         528
Asp Ser Phe Asp Trp Val Trp Ser Leu Glu Ser Gly Glu His Met Pro
                165                 170                 175

AAC AAA GCT CAG TTT TTA CAA GAA GCT TGG CGG GTA CTT AAA CCA GGT         576
Asn Lys Ala Gln Phe Leu Gln Glu Ala Trp Arg Val Leu Lys Pro Gly
            180                 185                 190

GGC CGT CTG ATT TTA GCG ACC TGG TGT CAT CGT CCC ATT GAT CCC GGC         624
Gly Arg Leu Ile Leu Ala Thr Trp Cys His Arg Pro Ile Asp Pro Gly
        195                 200                 205

AAT GGC CCC CTG ACT GCC GAT GAA CGT CGC CAT CTC CAA GCC ATC TAT         672
Asn Gly Pro Leu Thr Ala Asp Glu Arg Arg His Leu Gln Ala Ile Tyr
    210                 215                 220

GAC GTT TAC TGT TTG CCC TAT GTG GTT TCC CTG CCG GAC TAC GAG GCG         720
Asp Val Tyr Cys Leu Pro Tyr Val Val Ser Leu Pro Asp Tyr Glu Ala
225                 230                 235                 240

ATC GCC AGG GAA TGT GGG TTT GGG GAA ATT AAG ACT GCC GAT TGG TCA         768
Ile Ala Arg Glu Cys Gly Phe Gly Glu Ile Lys Thr Ala Asp Trp Ser
                245                 250                 255

GTG GCG GTG GCA CCT TTT TGG GAC CGG GTG ATT GAG TCT GCG TTC GAT         816
Val Ala Val Ala Pro Phe Trp Asp Arg Val Ile Glu Ser Ala Phe Asp
            260                 265                 270

CCC CGG GTG TTG TGG GCC TTG GGG CAA GCG GGG CCA AAA ATT ATC AAT         864
Pro Arg Val Leu Trp Ala Leu Gly Gln Ala Gly Pro Lys Ile Ile Asn
        275                 280                 285
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GCC GCC CTG TGT TTA CGA TTA ATG AAA TGG GGC TAT GAA CGG GGA TTA      912
Ala Ala Leu Cys Leu Arg Leu Met Lys Trp Gly Tyr Glu Arg Gly Leu
    290                 295                 300

GTG CGT TTT GGC TTA TTA ACG GGG ATA AAG CCT TTA GTT TGA             954
Val Arg Phe Gly Leu Leu Thr Gly Ile Lys Pro Leu Val  *
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 318 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Val Tyr His Val Arg Pro Lys His Ala Leu Phe Leu Ala Phe Tyr
 1               5                  10                  15

Cys Tyr Phe Ser Leu Leu Thr Met Ala Ser Ala Thr Ile Ala Ser Ala
            20                  25                  30

Asp Leu Tyr Glu Lys Ile Lys Asn Phe Tyr Asp Asp Ser Ser Gly Leu
        35                  40                  45

Trp Glu Asp Val Trp Gly Glu His Met His His Gly Tyr Tyr Gly Pro
    50                  55                  60

His Gly Thr Tyr Arg Ile Asp Arg Arg Gln Ala Gln Ile Asp Leu Ile
65                  70                  75                  80

Lys Glu Leu Leu Ala Trp Ala Val Pro Gln Asn Ser Ala Lys Pro Arg
                85                  90                  95

Lys Ile Leu Asp Leu Gly Cys Gly Ile Gly Gly Ser Ser Leu Tyr Leu
            100                 105                 110

Ala Gln Gln His Gln Ala Glu Val Met Gly Ala Ser Leu Ser Pro Val
        115                 120                 125

Gln Val Glu Arg Ala Gly Glu Arg Ala Arg Ala Leu Gly Leu Gly Ser
    130                 135                 140

Thr Cys Gln Phe Gln Val Ala Asn Ala Leu Asp Leu Pro Phe Ala Ser
145                 150                 155                 160

Asp Ser Phe Asp Trp Val Trp Ser Leu Glu Ser Gly Glu His Met Pro
                165                 170                 175

Asn Lys Ala Gln Phe Leu Gln Glu Ala Trp Arg Val Leu Lys Pro Gly
            180                 185                 190

Gly Arg Leu Ile Leu Ala Thr Trp Cys His Arg Pro Ile Asp Pro Gly
        195                 200                 205

Asn Gly Pro Leu Thr Ala Asp Glu Arg Arg His Leu Gln Ala Ile Tyr
    210                 215                 220

Asp Val Tyr Cys Leu Pro Tyr Val Val Ser Leu Pro Asp Tyr Glu Ala
225                 230                 235                 240

Ile Ala Arg Glu Cys Gly Phe Gly Glu Ile Lys Thr Ala Asp Trp Ser
                245                 250                 255
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Val Ala Val Ala Pro Phe Trp Asp Arg Val Ile Glu Ser Ala Phe Asp
          260                 265                 270

Pro Arg Val Leu Trp Ala Leu Gly Gln Ala Gly Pro Lys Ile Ile Asn
      275                 280                 285

Ala Ala Leu Cys Leu Arg Leu Met Lys Trp Gly Tyr Glu Arg Gly Leu
  290                 295                 300

Val Arg Phe Gly Leu Leu Thr Gly Ile Lys Pro Leu Val  *
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1790 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 207..1253

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
GCTCGCATGT TGTGTGGAAT TGTGAGCGGA TAACAATTTC ACACAGGAAA CAGCTATGAC      60

CATGATTACG CCAAGCTCTA ATACGACTCA CTATAGGGAA AGCTGGTACG CCTGCAGGTA     120

CCGGTCCGGA ATTCCCGGGT CGACCCACGC GTCCGCAAAT AATCCCTGAC TTCGTCACGT     180

TTCTTTGTAT CTCCAACGTC CAATAA ATG AAA GCA ACT CTA GCA GCA CCC TCT       233
                               Met Lys Ala Thr Leu Ala Ala Pro Ser
                                   320                 325

TCT CTC ACA AGC CTC CCT TAT CGA ACC AAC TCT TCT TTC GGC TCA AAG       281
Ser Leu Thr Ser Leu Pro Tyr Arg Thr Asn Ser Ser Phe Gly Ser Lys
        330                 335                 340

TCA TCG CTT CTC TTT CGG TCT CCA TCC TCC TCC TCC TCA GTC TCT ATG       329
Ser Ser Leu Leu Phe Arg Ser Pro Ser Ser Ser Ser Ser Val Ser Met
    345                 350                 355

ACG ACA ACG CGT GGA AAC GTG GCT GTG GCG GCT GCT GCT ACA TCC ACT       377
Thr Thr Thr Arg Gly Asn Val Ala Val Ala Ala Ala Ala Thr Ser Thr
360                 365                 370                 375

GAG GCG CTA AGA AAA GGA ATA GCG GAG TTC TAC AAT GAA ACT TCG GGT       425
Glu Ala Leu Arg Lys Gly Ile Ala Glu Phe Tyr Asn Glu Thr Ser Gly
                380                 385                 390

TTG TGG GAA GAG ATT TGG GGA GAT CAT ATG CAT CAT GGC TTT TAT GAC       473
Leu Trp Glu Glu Ile Trp Gly Asp His Met His His Gly Phe Tyr Asp
            395                 400                 405

CCT GAT TCT TCT GTT CAA CTT TCT GAT TCT GGT CAC AAG GAA GCT CAG       521
Pro Asp Ser Ser Val Gln Leu Ser Asp Ser Gly His Lys Glu Ala Gln
        410                 415                 420

ATC CGT ATG ATT GAA GAG TCT CTC CGT TTC GCC GGT GTT ACT GAT GAA       569
Ile Arg Met Ile Glu Glu Ser Leu Arg Phe Ala Gly Val Thr Asp Glu
    425                 430                 435
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GAG GAG GAG AAA AAG ATA AAG AAA GTA GTG GAT GTT GGG TGT GGG ATT      617
Glu Glu Glu Lys Lys Ile Lys Lys Val Val Asp Val Gly Cys Gly Ile
440                 445                 450                 455

GGA GGA AGC TCA AGA TAT CTT GCC TCT AAA TTT GGA GCT GAA TGC ATT      665
Gly Gly Ser Ser Arg Tyr Leu Ala Ser Lys Phe Gly Ala Glu Cys Ile
                460                 465                 470

GGC ATT ACT CTC AGC CCT GTT CAG GCC AAG AGA GCC AAT GAT CTC GCG      713
Gly Ile Thr Leu Ser Pro Val Gln Ala Lys Arg Ala Asn Asp Leu Ala
            475                 480                 485

GCT GCT CAA TCA CTC TCT CAT AAG GCT TCC TTC CAA GTT GCG GAT GCG      761
Ala Ala Gln Ser Leu Ser His Lys Ala Ser Phe Gln Val Ala Asp Ala
        490                 495                 500

TTG GAT CAG CCA TTC GAA GAT GGA AAA TTC GAT CTA GTG TGG TCG ATG      809
Leu Asp Gln Pro Phe Glu Asp Gly Lys Phe Asp Leu Val Trp Ser Met
    505                 510                 515

GAG AGT GGT GAG CAT ATG CCT GAC AAG GCC AAG TTT GTA AAA GAG TTG      857
Glu Ser Gly Glu His Met Pro Asp Lys Ala Lys Phe Val Lys Glu Leu
520                 525                 530                 535

GTA CGT GTG GCG GCT CCA GGA GGT AGG ATA ATA ATA GTG ACA TGG TGC      905
Val Arg Val Ala Ala Pro Gly Gly Arg Ile Ile Ile Val Thr Trp Cys
                540                 545                 550

CAT AGA AAT CTA TCT GCG GGG GAG GAA GCT TTG CAG CCG TGG GAG CAA      953
His Arg Asn Leu Ser Ala Gly Glu Glu Ala Leu Gln Pro Trp Glu Gln
            555                 560                 565

AAC ATC TTG GAC AAA ATC TGT AAG ACG TTC TAT CTC CCG GCT TGG TGC     1001
Asn Ile Leu Asp Lys Ile Cys Lys Thr Phe Tyr Leu Pro Ala Trp Cys
        570                 575                 580

TCC ACC GAT GAT TAT GTC AAC TTG CTT CAA TCC CAT TCT CTC CAG GAT     1049
Ser Thr Asp Asp Tyr Val Asn Leu Leu Gln Ser His Ser Leu Gln Asp
    585                 590                 595

ATT AAG TGT GCG GAT TGG TCA GAG AAC GTA GCT CCT TTC TGG CCT GCG     1097
Ile Lys Cys Ala Asp Trp Ser Glu Asn Val Ala Pro Phe Trp Pro Ala
600                 605                 610                 615

GTT ATA CGG ACT GCA TTA ACA TGG AAG GGC CTT GTG TCT CTG CTT CGT     1145
Val Ile Arg Thr Ala Leu Thr Trp Lys Gly Leu Val Ser Leu Leu Arg
                620                 625                 630

AGT GGT ATG AAA AGT ATT AAA GGA GCA TTG ACA ATG CCA TTG ATG ATT     1193
Ser Gly Met Lys Ser Ile Lys Gly Ala Leu Thr Met Pro Leu Met Ile
            635                 640                 645

GAA GGT TAC AAG AAA GGT GTC ATT AAG TTT GGT ATC ATC ACT TGC CAG     1241
Glu Gly Tyr Lys Lys Gly Val Ile Lys Phe Gly Ile Ile Thr Cys Gln
        650                 655                 660

AAG CCA CTC TAA GTCTAAAGCT ATACTAGGAG ATTCAATAAG ACTATAAGAG         1293
Lys Pro Leu  *
    665

TAGTGTCTCA TGTGAAAGCA TGAAATTCCT TAAAAACGTC AATGTTAAGC CTATGCTTCG   1353

TTATTTGTTT TAGATAAGTA TCATTTCACT CTTGTCTAAG GTAGTTTCTA TAAACAATAA   1413

ATACCATGAA TTAGCTCATG TTATCTGGTA AATTCTCGGA AGTGATTGTC ATGGATTAAC   1473

TCAAAAAAAA AAAAAAAAAA AGGGCGGCCG CTCTAGAGGA TCCAAGCTTA CGTACGCGTG   1533
```

CATGCGACGT CATAAGTCTA TCATACCGTC GACCTCGAGG GGGGCCCTAA ATTCAATTCA 1593

CTGGCCGTCG TTTTACAACG TCGTGACTGG GAAAACCCTG GCGTTACCCA ACTTAATCGC 1653

CTTGCAGCAC ATCCCCCTTT CGCCAGCTGG CGTAATAGCG AAGAGGCCCG CACCGATCGC 1713

CCTTCCCAAC AGTTGCGCAG CCTGAATGGC GAATGGGACG CGCCCTGTAG CGGCGCATTA 1773

AGCGCGGCGG GTGTGGT 1790

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 349 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Ala Thr Leu Ala Ala Pro Ser Ser Leu Thr Ser Leu Pro Tyr
1 5 10 15

Arg Thr Asn Ser Ser Phe Gly Ser Lys Ser Ser Leu Leu Phe Arg Ser
20 25 30

Pro Ser Ser Ser Ser Ser Val Ser Met Thr Thr Thr Arg Gly Asn Val
35 40 45

Ala Val Ala Ala Ala Ala Thr Ser Thr Glu Ala Leu Arg Lys Gly Ile
50 55 60

Ala Glu Phe Tyr Asn Glu Thr Ser Gly Leu Trp Glu Glu Ile Trp Gly
65 70 75 80

Asp His Met His His Gly Phe Tyr Asp Pro Asp Ser Ser Val Gln Leu
85 90 95

Ser Asp Ser Gly His Lys Glu Ala Gln Ile Arg Met Ile Glu Glu Ser
100 105 110

Leu Arg Phe Ala Gly Val Thr Asp Glu Glu Glu Glu Lys Lys Ile Lys
115 120 125

Lys Val Val Asp Val Gly Cys Gly Ile Gly Gly Ser Ser Arg Tyr Leu
130 135 140

Ala Ser Lys Phe Gly Ala Glu Cys Ile Gly Ile Thr Leu Ser Pro Val
145 150 155 160

Gln Ala Lys Arg Ala Asn Asp Leu Ala Ala Ala Gln Ser Leu Ser His
165 170 175

Lys Ala Ser Phe Gln Val Ala Asp Ala Leu Asp Gln Pro Phe Glu Asp
180 185 190

Gly Lys Phe Asp Leu Val Trp Ser Met Glu Ser Gly Glu His Met Pro
195 200 205

Asp Lys Ala Lys Phe Val Lys Glu Leu Val Arg Val Ala Ala Pro Gly
210 215 220

Gly Arg Ile Ile Ile Val Thr Trp Cys His Arg Asn Leu Ser Ala Gly
225 230 235 240

```
Glu Glu Ala Leu Gln Pro Trp Glu Gln Asn Ile Leu Asp Lys Ile Cys
                245                 250                 255

Lys Thr Phe Tyr Leu Pro Ala Trp Cys Ser Thr Asp Asp Tyr Val Asn
            260                 265                 270

Leu Leu Gln Ser His Ser Leu Gln Asp Ile Lys Cys Ala Asp Trp Ser
        275                 280                 285

Glu Asn Val Ala Pro Phe Trp Pro Ala Val Ile Arg Thr Ala Leu Thr
    290                 295                 300

Trp Lys Gly Leu Val Ser Leu Leu Arg Ser Gly Met Lys Ser Ile Lys
305                 310                 315                 320

Gly Ala Leu Thr Met Pro Leu Met Ile Glu Gly Tyr Lys Lys Gly Val
                325                 330                 335

Ile Lys Phe Gly Ile Ile Thr Cys Gln Lys Pro Leu  *
            340                 345
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACGGATCCAA AAATGCCTAT GGTTCATCAT CGGGG 35

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGGATCCTG TGGACTTCAA ACTAAAGGCT TTATC 35

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCTCATGATT TACCATGTTA GGCC 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGATCTCAAA CTAAAGGCTT TATC 24

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCATGCTGTG GCGGCTGCTG CTAC 24

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTCGACGCAT GCACGCGTAC GTAA 24

QBMAD\162725

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15137

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01H 5/00, 5/10; C07H 21/04; C12N 5/04, 15/63, 15/82
US CL :435/320.1; 536/23.6; 800/298
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1; 536/23.6; 800/298

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHIGEOKA et al. Isolation and Properties of γ-Tocopherol Methyltransferase in Euglena gracilis. Biochimica et Biophysica Acta. 1992, Vol. 1128, No. 2-3, pages 220-226, see entire document.	1-11
A	ISHIKO et al. Some Properties of γ-Tocopherol Methyltransferase Solubilized from Spinach Chloroplasts. Phytochemistry. May 1992, Vol. 31, No. 5, pages 1499-1500, see entire document.	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 OCTOBER 1998	Date of mailing of the international search report 12 NOV 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer THANDA WAI Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15137

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D'HARLINGUE et al. Plastid Enzymes of Terpenoid Biosynthesis: Purification and Characterization of γ-Tocopherol Methyltransferase From Capsicum Chromoplasts. Journal of Biological Chemistry. 05 December 1985, Vol. 260, No. 28, pages 15200-15203, see entire document.	1-11
A	MICHALOWSKI et al. Preliminary Characterization of S-Adenosylmethionine: Tocopherol Methyltransferase from Chloroplasts of Calendula officinalis Seedlings. Acta Biochimica Polonica. 1993, Vol. 40, No. 1, page 116-119, see entire document.	1-11
A	CAMARA et al. Enzymological Characterization of S-Adenosylmethionine γ-Tocopherol Methyltransferase From Capsicum Chromoplasts. Plant Physiology. April 1985, Vol. 77, No. 4, page 48, abstract number 257, see abstract.	1-11
A	SOLL et al. Localization and Synthesis of Prenylquinones in Isolated Outer and Inner Envelope Membranes from Spinach Chloroplasts. Archives of Biochemistry and Biophysics. April 1985, Vol. 238, No. 1, pages 290-299, see entire document.	1-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15137

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-11

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15137

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, AGRICOLA, BIOSIS, EMBASE, WPIDS

search terms: tocopherol, methyltransferase#, methyl transferase#

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. I

Group I, claims 1-11, drawn to an isolated DNA fragment comprising a gamma-tocopherol methyltransferase coding sequence, constructs comprising the DNA fragment, transgenic plants comprising the construct, and seeds from the plant, the first product, method of making, and method of using.

Group II, claim 12, drawn to oil from the seed of claim 11, the second product.

Group III, claims 13-14, drawn to transgenic plants altered to produce alpha-tocopherol as the most abundant tocopherol in the seeds of the plant and to seeds of the plant, the third product.

Group IV, claim 15, drawn to oil from the seeds of claim 14, the fourth product.

Group V, claims 16-18, drawn to transgenic plants carrying in its genome a foreign genetic construction comprising a gamma-tocopherol methyltransferase gene identified by SEQ ID NO:1 or SEQ ID NO:3, the fifth product.

Group VI, claim 19, drawn to oil from the seeds of claim 18, the sixth product.

Group VII, claim 20, drawn to transgenic plant seed containing alpha-tocopherol as the most abundant tocopherol present in the transgenic plant seed, the seventh product.

Group VIII, claim 21, drawn to oil from the seed of claim 20, the eight product.

Group IX, claims 22-24, drawn to transgenic plants having altered levels of tocopherols in its tissues, the plant comprising in its genome an inserted gamma-tocopherol methyltransferase coding sequence, the ninth product.

Group X, claim 25, drawn to a method of producing alpha-tocopherol by expressing a gamma-tocopherol methyltransferase coding sequence and reacting the product to produce alpha-tocopherol, the second method of using the first product.

The inventions listed as Groups I-X do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the products of Groups I-IX are distinct. The method of Group X is a second method of utilizing the product of Group I. PCT rule 13 does not provide for multiple products or multiple methods of using within a single application (37 CFR 1.475(d)).